APPLIED MICROBIOLOGY, July 1970, p. 69-71
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Vol. 20, No. 1
Printed in U.S.A.

Preservation of *Mycoplasma* Strains by Freezing in Liquid Nitrogen and by Lyophilization with Sucrose

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Received for publication 5 February 1970

Procedures for maintaining *Mycoplasma* strains are described. Liquid nitrogen storage provided an adequate means for keeping stock cultures stable. Over 74 strains of approximately 26 species have been preserved in this way, some for as long as 9 years. *Mycoplasma* strains can also be recovered satisfactorily from the freeze-dried state when the procedure described includes the use of 12% (v/v) sucrose as an additive. Fifteen strains representing 12 or more species were subjected to a freeze-drying program with and without sucrose added to concentrated cell suspensions in growth medium. Cell counts indicate improved survival with sucrose.

In 1960, the American Type Culture Collection (ATCC) initiated the development of a *Mycoplasma* collection to fill the need for a national repository and distribution center for these organisms of increasing importance in human and veterinary medicine. This step was made possible because it was found that mycoplasmas could be frozen and stored in liquid nitrogen refrigerators (−150 to −196 C) with little loss in viability. Such storage facilities by that time had become available at the ATCC. Furthermore, the collection was made possible by the willingness of specialists to collaborate by providing strains, guidance, and facilities for ATCC staff members to carry out certain phases of the work.

Since 1960, over 74 strains of *Mycoplasma* representing approximately 26 species have been accessioned into the ATCC (1).

Because of the expense and other impracticalities of distributing frozen cultures, freeze-drying was also investigated as a means of preserving mycoplasmas. Tully and Ruchman (8) reported recovering viable mycoplasmas from 20-year-old lyophilized cultures received from Sabin. D. G. ff. Edward (*personal communication*) has used lyophilization for preserving mycoplasmas since the 1950's. Morton described the methods he used for freeze-drying mycoplasmas at the American Society for Microbiology meetings in 1963. Andrews (2) mentioned the use of freeze-drying with glucose and sucrose as additives for preserving mycoplasmas. Our trials over the past 8 years have resulted in the development of the freeze-drying procedure reported here. Early attempts to freeze-dry mycoplasmas in a growth medium by using the double-vial "batch" system in use at the ATCC and detailed by Weiss (9) were relatively unsuccessful. However, we subsequently found that a single-ampoule manifold system similar to that described by Heckly et al. (5) showed promise. Furthermore, the addition to the suspending fluid of sucrose at a final concentration of 12% (v/v), previously found useful in this laboratory (4) and elsewhere (6, 7) as an additive in freeze-drying of other organisms, enhanced even more the recovery of freeze-dried mycoplasmas.

MATERIALS AND METHODS

Strains. The strains of *Mycoplasma* used in this study are recorded in Tables 1 and 2 and in the ATCC *Catalogue of Strains* (1). Cultures were incubated on a gyratory shaker rotating at 90 rev/min for 24 hr at 37 C in plastic centrifuge bottles (122 by 61.7 mm) containing broth (*see below*). Slow-growing species were incubated for as long as 96 hr.

Media. Most cultures were grown in Heart Infusion Broth (Difco) adjusted to *p*H 7.5 and enriched with 20% (v/v) heat-inactivated horse serum and 10% (v/v) of a 25% (w/v) fresh bakers' yeast extract. *M. orale* and *M. pneumoniae* were grown in PPLO Broth (Difco) enriched with 20% (v/v) unheated horse serum and 10% (v/v) of a 25% (w/v) fresh bakers' yeast extract. *M. arginini* was grown in the medium of Barile et al. (3).

Harvesting procedure. Cultures were harvested by centrifugation at 13,200 × *g* for 20 min in a Sorvall automatic superspeed refrigerated centrifuge (RC-2).

After the supernatant was decanted, the cells were resuspended in fresh, sterile broth-culture medium to produce a concentration of cells 30 times greater than that in the original culture. Purity of the culture was verified by inoculating the concentrated material on an agar gel of the recommended broth medium, on 4% (v/v) sheep blood-agar, in fluid thioglycolate medium, and by examination of a Gram-stained smear.

Freezing procedure. The concentrated culture was diluted 1:1 with 20% (v/v) sterile glycerol in distilled water, mixed, and dispensed into cotton-plugged 1.2-ml Cryules (catalogue no. 12742, Wheaton Glass Co., Millville, N.J.) which were then hermetically sealed. Ampoules were cooled at a rate of 1 C/min to −40 C by means of a BF-3-2 freezer (Linde Co., Tonawanda, N.Y.) and thereafter at an uncontrolled rate to the temperature of liquid nitrogen. They were stored in a liquid nitrogen refrigerator at −150 to −196 C.

Freeze-drying procedure. The concentrated culture suspension was divided into two equal portions. Fresh culture medium was added to the first portion in a ratio of 1:1 (v/v). The second portion was mixed 1:1 with a 24% (w/v) sterile aqueous sucrose solution. Amounts of the respective cell suspensions (0.2 ml)

TABLE 1. *Preservation of Mycoplasma strains in liquid nitrogen*

Mycoplasma species	Strain	ATCC no.	Colony forming units/ml[a] before freezing	Colony forming units/ml[a] after freezing	Survival (%)
M. agalactiae var. *bovis*	Donetta	25523	8.3×10^9	8.7×10^9	>100.0
M. anatis	1340	25524	5.7×10^9	5.1×10^9	89.5
M. arginini	G 230	23838	2.6×10^9	1.3×10^9	50.0
M. arthritidis	H 606	13988	2.0×10^9	1.9×10^9	95.0
M. bovimastitidis	01	25025	3.7×10^9	7.0×10^9	>100.0
M. felis	CO	23391	1.4×10^7	1.8×10^6	12.9
M. hominis	PG 21	23114	1.5×10^8	5.3×10^7	35.3
M. hyorhinis	PG 29	25026	3.4×10^{10}	1.4×10^{10}	41.2
M. hyorhinis	BTS 7	17981	3.5×10^9	1.7×10^9	48.6
M. laidlawii	Laidlaw A	14089	2.1×10^{10}	6.5×10^9	30.9
M. leonis	LL	25528	9.0×10^9	1.0×10^{10}	>100.0
M. meleagridis	17529	25294	4.7×10^9	2.3×10^9	48.9
M. orale type 1	CH 19299	23714	5.6×10^{10}	4.4×10^9	7.9
M. pneumoniae	Mac	15492	1.9×10^8	2.0×10^8	>100.0
Mycoplasma sp.	67–166	23243	4.4×10^9	2.0×10^9	45.4

[a] Average of triplicate determinations on contents of each of two ampoules.

TABLE 2. *Effects of adding sucrose to the suspending medium on the freeze-drying of mycoplasma strains*

Mycoplasma species	Strain	ATCC no.	Suspending medium without sucrose			Suspending medium with sucrose		
			Colony forming units/ml[a] before freeze-drying	Colony forming units/ml[a] after freeze-drying	Survival (%)	Colony forming units/ml[a] before freeze-drying	Colony forming units/ml[a] after freeze-drying	Survival (%)
M. agalactiae var. *bovis*	Donetta	25523	6.8×10^9	6.2×10^8	9.1	6.8×10^9	1.7×10^9	25.0
M. anatis	1340	25524	5.3×10^9	4.7×10^8	8.9	6.8×10^9	3.5×10^8	5.1
M. arginini	G 230	23838	2.6×10^9	1.5×10^8	5.8	2.8×10^9	4.8×10^8	17.1
M. arthritidis	H 606	13988	9.0×10^{10}	1.2×10^9	1.3	6.0×10^{10}	2.5×10^9	4.2
M. bovimastitidis	01	25025	2.6×10^9	2.7×10^7	1.0	2.4×10^9	1.4×10^7	0.6
M. felis	CO	23391	1.4×10^7	0	0.0	4.8×10^7	3.6×10^5	0.8
M. hominis	PG 21	23114	1.7×10^9	1.2×10^6	0.1	3.5×10^9	6.7×10^8	19.1
M. hyorhinis	PG 29	25026	1.5×10^{10}	1.3×10^9	8.7	1.3×10^{10}	1.0×10^{10}	76.9
M. hyorhinis	BTS 7	17981	3.3×10^9	1.7×10^7	0.5	4.1×10^9	2.0×10^9	48.8
M. laidlawii	Laidlaw A	14089	1.4×10^{10}	1.6×10^9	11.4	1.3×10^{10}	3.0×10^9	23.1
M. leonis	LL	25528	9.0×10^9	1.4×10^8	1.6	1.0×10^{10}	3.2×10^8	3.2
M. meleagridis	17529	25294	4.7×10^9	2.4×10^9	51.1	4.7×10^9	2.9×10^9	61.7
M. orale type 1	CH 19299	23714	2.9×10^9	5.0×10^7	1.7	3.4×10^9	2.6×10^8	7.6
M. pneumoniae	Mac	15492	1.5×10^8	7.7×10^6	5.1	2.0×10^8	1.8×10^8	90.0
Mycoplasma sp.	67–166	23243	4.4×10^9	4.5×10^8	10.2	4.4×10^9	2.9×10^8	6.6

[a] Average of triplicate determinations on contents of each of two ampoules.

were dispensed into sterile cotton-plugged 1.0-ml teardrop ampoules (catalogue no. 4020, Bellco Glass Co., Inc., Vineland, N.J.). The cell suspensions were frozen by agitating the ampoules in a dry ice-ethylene glycol-monoethyl ether bath (freezing rate, approximately 100 C/min). Ampoules containing the frozen cell suspensions and still immersed in the freezing bath were then attached to a 30-port manifold connected in series by using amber latex tubing to a −79 C (dry ice-ethylene glycol-monoethyl ether) condenser and a vacuum pump as described by Heckly (5). After a vacuum of 50 μm of Hg or less was attained (as determined with a McLeod gauge, Stokes Machine Co., Philadelphia, Pa.), the dry ice was allowed to sublime, and the bath was permitted to reach room temperature at an uncontrolled rate (during a period of approximately 3 hr). The vacuum was maintained throughout the drying (ca. 18 hr). Thereafter, the ampoules were sealed under vacuum at a point just below the cotton plug. Ampoules were stored at −70 C.

Determination of viable cell counts. Prior to processing, two portions (0.1 ml) of concentrated culture were diluted 10-fold serially with appropriate growth medium. Samples from the dilution tubes were plated in triplicate. For postfreezing determinations, two ampoules of each strain were removed from frozen storage and thawed rapidly by swirling in a 37 C water bath until the contents were visibly thawed; they were then diluted and plated as above. The total viable cell counts of freeze-dried cultures were determined by rehydrating each culture in duplicate with 0.2 ml of broth, and then diluting and plating.

RESULTS

Of 15 strains of *Mycoplasma* frozen and stored in liquid nitrogen, all showed good recovery (Table 1). Some 20 strains other than those listed in Table 1 have been frozen and have remained stable for 3 years in liquid nitrogen storage. Regarding the recovery of mycoplasmas from freeze-drying, the effect of adding sucrose to the medium prior to processing is shown in Table 2.

In initial trials without sucrose, recovery of *M. felis* was unsuccessful and that of *M. hominis* was poor. By using sucrose as a protective additive, the colony counts after lyophilization were appreciably improved. Because of the improvement with these strains, sucrose has been incorporated as a routine additive to all *Mycoplasma* strains now lyophilized by the ATCC.

There was variation between the strains in their response to the addition of sucrose (Table 2). Whereas *M. felis*, *M. hominis*, and *M. pneumoniae* showed markedly improved recovery, *M. anatis*, *M. bovimastitidis*, and *Mycoplasma* species 67–166 were apparently unaffected. The rest of the strains showed moderate improvement.

DISCUSSION

The strains of *Mycoplasma* tested showed various degrees of sensitivity to freezing. For example, *M. orale* ATCC 23714 showed 7.9% survival, and *M. arthritidis* ATCC 13988 showed 95% survival. The even higher survival rates of strain numbers ATCC 25523, 25025, 25528, and 15492 can be explained at this time only by disaggregation of the cells in the freeze-thaw procedure. Of the procedures for freeze-drying mycoplasmas tried at the ATCC, the method described above not only gave the best recoveries in terms of total cell counts but also was the only one that permitted the recovery of all strains submitted to the procedure. Pending controlled experiments to determine the effect of various storage temperatures on the stability of freeze-dried mycoplasmas, they are being stored at −70 C in an electric ULT-659 refrigerator (Revco Manufacturing Co., Deerfield, Mich.).

In general, better recovery was obtained from cultures stored in liquid nitrogen than from those freeze-dried.

ACKNOWLEDGMENTS

The American Type Culture Collection is grateful to Ruth G. Wittler for her major role in establishing and guiding the collection of *Mycoplasma* strains. We also thank the Walter Reed Army Institute of Research for providing facilities for propagation of strains for the collection.

Funds for preservation of this collection were provided by Public Health Service grant FR 00117 from the Division of Research Facilities and Resources.

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